

CLAIMS

We claim:

1. A method of domain specific gene evolution of a target nucleic acid sequence encoding an amino acid sequence of interest, said method comprising providing a plurality of pairs of single-stranded targeting polynucleotides which are substantially complementary to each other and each comprising a homology clamp that substantially corresponds to or is substantially complementary to a predetermined sequence of said target nucleic acid sequence encoding a domain of said protein, said plurality of pairs comprising a library of mismatches between said targeting polynucleotides and said sequence and a recombinase, to form a library of altered target nucleic acid sequences.
- 10 2. A method according to claim 1, further comprising simultaneously or successively providing a second plurality of pairs of single-stranded targeting polynucleotides, which are substantially complementary to each other and are not substantially complementary to said first plurality of polynucleotides and each comprising a second homology clamp, that substantially corresponds to or is substantially complementary to a second predetermined sequence of said target nucleic acid sequence encoding a second domain of said protein, said second plurality of pairs comprising a library of mismatches between said targeting polynucleotides and said second sequence and a recombinase, to form a library of altered target nucleic acid sequences.
3. A method of domain specific gene evolution comprising:
 - a) combining a target nucleic acid encoding an amino acid sequence of interest with a pair of single-stranded targeting polynucleotides which are substantially complementary to each other and each comprising a homology clamp that substantially corresponds to or is substantially complementary to a predetermined sequence of said nucleic acid encoding a domain of said protein, and a recombinase, to form a recombination intermediate;
 - b) contacting said intermediate with a single-strand exonuclease or junction-specific nuclease to form a nicked or open ended target nucleic acid; and
 - c) reassembling and recombining said nicked or open ended target nucleic acid to produce a library of altered target nucleic acids.
- 25 4. A method according to claim 3 further comprising:
 - d) simultaneously or successively combining said target nucleic acid encoding said amino acid sequence of interest with a second pair of single-stranded targeting polynucleotides which are substantially complementary to each other and are not substantially complementary to said first pair of polynucleotides and each comprising a homology clamp that substantially corresponds to or is substantially complementary to a second predetermined sequence of said nucleic acid encoding a second domain of said protein, and a recombinase, to form a recombination intermediate;
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e) contacting said intermediate with a single-strand exonuclease or junction-specific nuclease to form a nicked or open ended target nucleic acid; and

f) reassembling and recombining said nicked or open ended target nucleic acid to produce a library of altered target nucleic acids.

5 5. A method of generating a pool of variant nucleic acid sequences of a pre-selected target nucleic acid sequence in an extrachromosomal sequence, said method comprising:

10 a) adding to said extrachromosomal sequence at least one recombinase and a plurality of pairs of single-stranded targeting polynucleotides which are substantially complementary to each other and each comprising a homology clamp that substantially corresponds to or is substantially complementary to a preselected target nucleic acid sequence, said plurality of pairs comprising a library of mismatches between said targeting polynucleotide and said target nucleic acid sequence, to form a library of altered extrachromosomal sequences; and

15 b) repeating step a) on said library of altered extrachromosomal sequences.

6. A method according to claim 5 further comprising:

20 d) adding simultaneously or successively to said extrachromosomal sequence at least one recombinase and a second plurality of pairs of single-stranded targeting polynucleotides which are substantially complementary to each other and are not substantially complementary to said first plurality of polynucleotides and each comprising a homology clamp that substantially corresponds to or is substantially complementary to a second preselected target nucleic acid sequence, said second plurality of pairs comprising a library of mismatches between said targeting polynucleotide and said second target nucleic acid sequence, to form a library of altered extrachromosomal sequences; and

25 e) repeating step d) on said library of altered extrachromosomal sequences.

7. A method of generating a pool of variant nucleic acid sequences of a pre-selected target nucleic acid sequence in an chromosomal sequence, said method comprising:

30 a) adding to said chromosomal sequence at least one recombinase and a plurality of pairs of single-stranded targeting polynucleotides which are substantially complementary to each other and each comprising a homology clamp that substantially corresponds to or is substantially complementary to a preselected target nucleic acid sequence, said plurality of pairs comprising a library of mismatches between said targeting polynucleotide and said target nucleic acid sequence, to form a library of altered chromosomal sequences; and

35 b) repeating step a) on said library of altered extrachromosomal sequences.

8. A method according to claim 7 further comprising:

35 d) adding simultaneously or successively to said chromosomal sequence at least one recombinase and a second plurality of pairs of single-stranded targeting polynucleotides which

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are substantially complementary to each other and are not substantially complementary to said first plurality of polynucleotides and each comprising a homology clamp that substantially corresponds to or is substantially complementary to a second preselected target nucleic acid sequence, said second plurality of pairs comprising a library of mismatches between said targeting polynucleotide and said second target nucleic acid sequence, to form a library of altered chromosomal sequences; and

e) repeating step d) on said library of altered chromosomal sequences.

9. A method according to claim 1, 2, 3, or 4 further comprising repeating said method on said library of altered target nucleic acid sequences.

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10. A method according to claim 1, 2, 3, 4, 5, 6, 7, or 8 further comprising introducing said library of altered target nucleic acid sequences into cells to form a cellular library comprising variant nucleic acid sequences.

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11. A method according to claim 10 further comprising expressing said library of altered target nucleic acid sequences to generate a pool of variant amino acid sequences.

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12. A method according to claim 10 or 11 further comprising selecting a cell comprising an altered target nucleic acid sequence having a desired activity.

13. A method according to claim 10 or 11 further comprising selecting a cell comprising an altered target nucleic acid sequence having a desired phenotype.

14. A method according to claim 11 further comprising secreting said pool of variant amino acid sequences.

15. A method according to claim 10, 11, 12, or 13 wherein said recombinase is removed prior to said introducing.

16. A method according to claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 wherein said cells are eukaryotic.

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17. A method according to claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 wherein said cells are prokaryotic.

18. A method according to claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or 17 wherein said targeting polynucleotides are coated with said recombinase.

19. A method according to claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 wherein said recombinase is a species of prokaryotic recombinase.

20. A method according to claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 wherein said recombinase is a species of eukaryotic recombinase.

5 21. A method according to claim 11, wherein the variant amino acid sequences comprise a plurality of amino acid substitutions.

22. A method according to claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 wherein at least one of said complementary single stranded nucleic acids further comprise a chemical substituent.

10 23. A method according to claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or 22 wherein the target amino acid sequence comprises a complementary determining region.

24. A method according to claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, or 23 wherein said target nucleic acid sequence comprises an expression vector.